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Production of mycophenolic acid by *Penicillium brevicompactum*—A comparison of two methods of optimization

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ABSTRACT

Production of mycophenolic acid (MPA) by submerged fermentation using the microfungus *Penicillium brevicompactum* MTCC 8010 is reported here. Screening experiments were used to identify: the suitable media composition; the optimal initial pH; and the optimal incubation temperature to maximize the production of MPA in batch cultures. The initial concentrations of the selected sources of carbon (glucose), nitrogen (peptone) and the precursors (methionine, glycine) were then optimized by: (1) one-at-a-time variation of factors; and (2) a central composite design (CCD) of experiments, in a 12-day batch culture at an initial pH of 5.0, an incubation temperature of 25 °C, and an agitation speed of 200 rpm. The medium optimized using the one-at-a-time variation yielded a peak MPA titer of 1232 ± 90 mg/L. The medium optimized by the CCD method yielded a 40% higher MPA titer of 1737 ± 55 mg/L. The latter value was nearly 9-fold greater than the titer achieved prior to optimization.

1. Introduction

Mycophenolic acid (6-(4-hydroxy-6-methoxy-7-methyl-3oxophthalanyl)-4-methyl-4-hexenic acid, $C_{17}H_{20}O_6$, MPA) is a fungal secondary metabolite that was first isolated in 1893 as an antibiotic against *Bacillus anthracis* [1,2]. MPA is produced by many *Penicillium* species [3–8] as well as other fungi [6,9]. MPA and its derivatives are commercially used as frontline immunosuppressive agents to prevent rejection of transplant organs [10–12]. Commercial immunosuppressants based on MPA include CellCept (mycophenolate mofetil; Roche) and Myfortic (mycophenolate sodium; Novartis). In 2014, the sales of Myfortic were around US$ 543 million [13]. Similarly, in 2013 US$ 938 million worth of CellCept was sold [14]. Mycophenolate mofetil in combination with cyclosporine A (CyA) has been shown to reduce the incidence of graft rejection to 17% compared to a 60% rejection when CyA was used alone.

Here we report on the production of mycophenolic acid (MPA) by *Penicillium brevicompactum* MTCC 8010 (Institute of Microbial Technology, Chandigarh). The batch fermentation conditions are optimized to maximize the production of MPA. Two optimization approaches are compared: (1) the conventional one-at-a-time variation of factors; and (2) a simultaneous variation of the multiple factors based on a statistically robust central composite experiment design.

The relevant aspects of biosynthesis of MPA by *P. brevicompactum* have been discussed in the literature [1,15,16]. The chemistry, synthesis and modifications of MPA for improving the biological activity have been previously reviewed [17]. In submerged batch fermentations, low-intensity pulsed ultrasound has enhanced the production of MPA by *P. brevicompactum* [18]. Production in this fungus may also be influenced by the intensity and wavelength of the prevailing light [19], although most commercial submerged fermentations are inevitably conducted in the dark. Production of MPA by solid-state fermentation has been extensively reported in literature [7,20,21], but solid state fermentations are generally not used for the commercial production of medicinal products.

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2. Materials and methods

2.1. Microorganism and inoculum preparation

The microfungus *Penicillium brevicompactum* MTCC 8010 used in this work was obtained from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained aseptically on potato dextrose agar (PDA) slants. A spore suspension was used as inoculum. The spores scarped from the surface of a slant were transferred to PDA petri plates and incubated at 25 °C for 3-5 days. The spores from the plates were harvested aseptically with a sterile loop and dispersed in sterile distilled water to obtain a concentration of 10^7 spores per milliliter. A hemocytometer was used for the spore counts.

2.2. Media

The basal medium had the following composition (g/L): glucose 10, glycine 2, methionine 0.5, KH_2PO_4 2, $MgSO_4{\cdot}7H_2O$ 1, and 1 mL/L of a trace element solution. The latter contained the following (g/L): $FeSO_4{\cdot}7H_2O$ 2.2, $CuSO_4{\cdot}5H_2O$ 0.3, $ZnSO_4{\cdot}7H_2O$ 2.4, $MnSO_4{\cdot}4H_2O$ 0.16, and $(NH_4)_2MoO_4$ 0.2. The media components except glycine, methionine and trace element solution, were autoclaved separately at 121 °C for 15 min after the pH had been adjusted to 6.0 with 2 M HCl or 2 M NaOH solutions. Solutions of glycine, methionine and the trace elements were sterilized using a sterile 0.2 μm membrane filter (Millipore; www.emdmillipore.com).

2.3. Fermentations

Batch fermentations were carried out in 250 mL shake flasks each containing 50 mL of the culture medium. The medium always contained the following components (per L): $MgSO_4{\cdot}7H_2O$ 1 and the trace element solution (1 mL) specified earlier. This medium was supplemented with the required concentrations of the specified carbon source, the nitrogen source, the phosphate source and the potential precursors. The initial pH was adjusted to the required value. This medium (50 mL) was inoculated with 0.5 mL of the above specified spore suspension. Incubation occurred at the specified temperature. All fermentations were lasted for 12 days. The agitation speed of the platform shaker was 200 rpm. All fermentations were run in duplicate.

2.4. Analytical methods

2.4.1. Mycophenolic acid concentration

The MPA concentration was measured in the culture supernatant at the end of fermentation. The pH of the filtered supernatant was adjusted to 2.0 with 1 M HCl. This supernatant (50 mL) was extracted twice with ethyl acetate (100 mL for each extraction). The total duration of extraction was 30 min. The organic phase was recovered, pooled and evaporated at reduced pressure in a rotary evaporator (Rotavapor, R-200; www.buchi.com). The residue was dissolved in 1 mL of methanol, filtered through a 0.45 μm filter and measured via high performance liquid chromatography (HPLC) (Waters Alliance HPLC; www.waters.com). A C_{18} column (Symmetry® C_{18}, 5 μm, 4.6 × 250 mm) was used at 40 °C. The mobile phase was water and acetonitrile (50: 50 by vol), pH 3.0, at a flow rate of 0.5 mL/min. The injection volume was 10 μL. A photodiode array detector was used at a wavelength of 220 nm [22].

A standard curve (data not shown) was prepared using HPLC-grade authentic MPA (Sigma-Aldrich, USA). A HPLC chromatogram of MPA is shown in Fig. S1. A fresh curve was made each time a batch of the samples was analyzed. The stock solution of MPA (1 mg/mL) was prepared in methanol and stored at −20 °C. When required, the solution was serially diluted with methanol to provide MPA concentrations in the range of 2.5–125 μg/mL. All measurements of MPA were in triplicate.

2.4.2. Glucose concentration

The glucose concentration was measured in the culture supernatant as reducing sugars by the dinitrosalicylic acid (DNS) method [23]. A suitably diluted sample (1 mL) was mixed with 1 mL of the DNS reagent, held at 90 °C for 10 min, and cooled. The absorbance was measured at 540 nm against a blank of deionized water treated the same way as the sample. The measured absorbance and the dilution factor were used to estimate the reducing sugar concentration by comparing with a calibration curve made using dilutions of a standard aqueous solution of glucose.

2.4.3. Cell mass concentration

The fungal biomass in the fermentation broth was quantified by the dry cell weight method. A 50 mL sample of the fermentation broth was filtered through Grade 50 cheese cloth (purchased from the local market). The biomass was washed with 50 mL of water and the resulting cake was dried at 60–65 °C for at least 48 h [7,24]. The cake was cooled in a desiccator and weighed.

2.5. Optimization of fermentation medium using one-factor-at-a-time method

2.5.1. Carbon sources

The specified carbon sources (glucose, sucrose, fructose, maltose, lactose, glycerol, xylose, sorbitol, mannitol, starch and carboxymethyl cellulose) were studied at an initial concentration of 10 g/L. The carbon source that proved to be the best was examined further at various initial concentrations in the range 10–100 g/L. The other components of the production medium were held at the following initial concentrations (g/L): tryptone 5, glycine 2, methionine 0.5, KH_2PO_4 2, and $MgSO_4{\cdot}7H_2O$ 1. All media were supplemented with the earlier specified solution of trace elements (1 mL/L). The fermentation conditions were as follows: 27 °C; an initial pH of 6.0; an agitation speed of 200 rpm; and a fermentation duration of 12 days. All fermentations were carried out in duplicate.

2.5.2. Nitrogen sources

In the screening of the nitrogen sources, the carbon source was always glucose at an initial concentration of 60 g/L. The various organic and inorganic nitrogen sources (urea, ammonium nitrate, tryptone, peptone and asparagine) were screened individually as nitrogen sources. The initial concentration of a nitrogen source was always 10 g/L. Peptone was further examined at initial concentrations in the range of 10–40 g/L. The other components of the medium were held at the following initial concentrations (g/L): glycine 2, methionine 0.5, KH_2PO_4 2, and $MgSO_4{\cdot}7H_2O$ 1. All media were supplemented with the earlier specified solution of trace elements (1 mL/L). The fermentation conditions were as follows: 27 °C; an initial pH of 6.0; an agitation speed of 200 rpm; and a fermentation duration of 12 days. All fermentations were carried out in duplicate.

2.5.3. Phosphorous sources

Four different phosphate sources (KH_2PO_4, K_2HPO_4, NaH_2PO_4 and Na_2HPO_4) were screened individually at an initial concentration of 6 g/L. The other components of the medium were held at the following initial concentrations (g/L): glucose 60, peptone 20, glycine 2, methionine 0.5, and $MgSO_4{\cdot}7H_2O$ 1. All media were supplemented with the earlier specified solution of trace elements